Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1867–1871, March 1993
Cell Biology

Neural expression and chromosomal mapping of Neu differentiation factor to 8p12–p21

(polypeptide growth factor/receptor tyrosine kinase/*erbB-2*/nervous system)

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Communicated by Michael Sela, December 3, 1992 (received for review October 13, 1992)

ABSTRACT **Neu differentiation factor (NDF/heregulin) is a 44-kDa glycoprotein that interacts with the Neu/ErbB-2 receptor tyrosine kinase to increase its phosphorylation on tyrosine residues. *In vitro* NDF promotes differentiation of certain mammary tumor cell lines to milk-producing cells. As a first step toward understanding the physiological role of NDF, we performed *in situ* hybridization analyses to determine mRNA distribution in the mouse embryo and to map the gene to human karyotypes. In 14.5-day-postcoitum mouse embryos, NDF expression is confined predominantly to the central and peripheral nervous system, including the neuroepithelium that lines the lateral ventricles of the brain, the ventral horn of the spinal cord, and the intestinal as well as dorsal root ganglia. Other tissues that contain NDF transcripts are the adrenal gland, liver, and distinct cell layers of the dermis and germinal ridge. *In situ* hybridization of a ^{3}H-labeled probe to human metaphase spreads localized the NDF gene to the short arm of chromosome 8 at bands p12–p21.**

Receptor tyrosine kinases are transmembrane glycoproteins that transmit growth-regulatory signals through binding of polypeptide factors to their extracellular domains that consequently activate the intrinsic tyrosine kinase function of the receptor (reviewed in ref. 1). Epidermal growth factor (EGF) receptor is a prototypic tyrosine kinase that binds many distinct growth factors that contain an EGF-like motif (2). The *neu/erbB-2* protooncogene encodes a molecule that is closely related to EGF receptor, yet it binds none of the ligands of this receptor (3). Originally, *neu* was identified as a dominant transforming gene in tumors of the peripheral nervous system that were induced by transplacental treatment of rat embryos with *N*-ethylnitrosourea (4, 5). Interestingly, the period of susceptibility of *neu* to carcinogenesis—namely, midgestation—correlates with the timing of its expression in the nervous system (6). This may identify a developmental stage in which activation of Neu, probably by its endogenous ligand, has a critical physiological role.

The existence of a Neu-specific ligand was inferred from experiments with chimeric proteins between Neu and the EGF receptor (7–11) and agonistic monoclonal antibodies to the 185-kDa *neu* gene product, p185^{neu} (12–14). Attempts to isolate an activity that corresponded to a ligand of Neu identified *ras*-transformed fibroblasts as a source of the candidate ligand (15). Partial purification of the activity showed that it corresponded to a heat-stable and disulfide-containing protein that behaved chromatographically as a 35-kDa molecule (16). Complete purification yielded a 44-kDa glycoprotein that stimulated tyrosine phosphorylation of p185^{neu} in mammary tumor cells and induced these cells to differentiate to milk-producing mature cells (17). On the basis of this observation, the factor was named Neu differentiation factor (NDF). Molecular cloning of the NDF transcript showed that the precursor of the soluble factor is a membrane protein that contains a single transmembrane domain, a large cytoplasmic tail, and an extracellular part that carries an EGF-like domain and an immunoglobulin motif (18). Several human homologs of NDF, collectively termed heregulins, were independently identified in a human mammary tumor cell line, and a recombinant heregulin molecule that contained only the EGF-like domain was found to be growth-stimulatory for mammary tumor cells, but at high ligand concentrations it was inhibitory (19).

The physiological role of NDF has not yet been directly addressed. The gene is transcriptionally activated by transformation with the *ras* oncogene (18), and it is normally expressed in many tissues, including brain, ovary, testis, prostate, and lung (18, 19). The present studies were aimed to obtain additional clues to the function of NDF through analysis of its spatial pattern of expression in a mammalian embryo and by determination of its chromosomal localization.

MATERIALS AND METHODS

Embryos. C57BL/6J mice were used. The time of gestation was calculated by considering the morning after mating as day 0.5 post coitum (0.5 dpc) (20). All embryos were prefixed for 15 hr in 4% paraformaldehyde at 4°C.

Preparation of RNA Probes for Mouse NDF. Total cytoplasmic RNA from mouse cortex was isolated by the guanidinium thiocyanate/cesium chloride method (21). NDF-specific sequences were amplified by using oligonucleotides: 5′-TGAAGAGCCAGGAGTCAGCTGCAGG-3′ (5′ primer) and 5′-GGCTCGAGACTCTGAGGACACATAGG-3′ (3′ primer). The first strand of cDNA was synthesized at 37°C with reverse transcriptase (5 units, Promega), oligo(dT) (20 pmol) as a primer, and 10 μg of RNA. Polymerase chain reaction was then performed with both oligonucleotides (50 pmol of each) for 35 cycles each consisting of 1.5 min of denaturation at 95°C, 2 min of annealing at 56°C, and 3 min of elongation at 72°C. For cloning, the amplified DNA was ethanol-precipitated, air-dried, and resuspended in TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA) before being digested with *Pst* I and *Xho* I. Both restriction sites were incorporated by the synthetic oligonucleotides that were used for DNA amplification. The resulting fragment of 0.3 kb was inserted into pBluescript (Stratagene). Nucleotide sequence analysis of the insert confirmed that it was >90% identical to the corresponding portion of rat NDF clone 44 (18) that lies

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Abbreviations: NDF, Neu differentiation factor; EGF, epidermal growth factor; dps, day(s) post coitum.

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between nucleotides 195 and 530. T3 and T7 RNA polymerases were used to generate [α-[^{35}S]thio]UTP-labeled sense and antisense transcripts that were used as probes. This fragment of the NDF transcript was chosen as the hybridization probe because it was included in all the alternative forms of NDF mRNA (ref. 19 and unpublished observations).

Preparation of a DNA probe for Human NDF. A cDNA clone of human NDF was isolated from a cDNA expression library that was prepared with template mRNA isolated from a human cDNA library. The length of the insert cDNA was 1.8 kb, and nucleotide sequence analysis indicated that it corresponded to the whole cytoplasmic and transmembrane domains of NDF (18, 19). Agarose gel-purified DNA was labeled with a mixture of all four [^{3}H]dNTPs (0.75 nmol of each, Amersham) by the random primer technique (22) with a kit from Boehringer Mannheim. The [^{3}H]dNTPs were first lyophilized and then suspended in water (0.04 ml). NDF DNA (200 ng) was denatured at 100°C, cooled on ice, and then mixed with the nucleotides in a reaction buffer that contained also bovine serum albumin (0.5 mg/ml) and Klenow DNA polymerase. The reaction mixture was incubated at 22°C for 18 hr, after which salmon sperm DNA was added to 2 mg/ml and free nucleotides were removed by chromatography on a Sephadex G-50 column (Pharmacia). The specific activity obtained was 10^9 cpm/μg of DNA.

***In Situ* Hybridization to Mouse Embryos.** Embedding, sectioning, postfixation, and hybridization were performed as described (20). Cryostat sections (7–10 μm) were prepared. Post-hybridization washing was performed in 2× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0)/50% formamide/0.1 M dithiothreitol at 65°C for 30 min (high-stringency wash). This was followed by digestion with RNase and by a repeated high-stringency wash. For autoradiography, Kodak NTB/2 emulsion was used and the slides were exposed for 3–5 weeks. Finally, the sections were stained in Giemsa stain. The slides were photographed either in a Wild/Leitz microscope for low magnifications or in a Zeiss photomicroscope for higher magnifications. Kodak Technical Pan negative film was used for bright-field, and Kodak T Max 400 for dark-field microscopy.

Chromosome Preparation. Metaphase spreads were obtained from phytohemagglutin (PHA)-stimulated peripheral blood lymphocytes from a normal male by a modification of the previously described bromodeoxyuridine (BrdUrd) synchronization technique (23). Ten drops of whole blood were cultured for 96 hr at 37°C in 10 ml of McCoy's 5A medium with 15% fetal bovine serum (HyClone) in the presence of 0.1 ml of PHA (Wellcome). Cells were synchronized by the addition of BrdUrd (25 μg/ml; Sigma) and incubated for 16 hr. The cells were then washed twice with medium (without serum) and cultured for 6 hr in fresh medium that contained 10 μM thymidine (Sigma). Colcemid (GIBCO-BRL) was added (50 ng/ml) 10 min prior to harvesting. The lymphocytes were treated for 15 min with a hypotonic solution (75 mM KCl), and fixed by three changes of methanol/acetic acid (3:1, vol/vol). Three drops of the cell suspension were dropped on a slide from a height of 30 cm and the slides were air-dried. Slides were kept at −20°C and were thawed on the day of hybridization.

***In Situ* Hybridization to Human Chromosomes.** The procedure was based on a published method (24). Slides were treated for 1 hr at 37°C with 150 μl of pancreatic RNase A (100 μg/ml in 2× SSC). They were then rinsed in four changes of 2× SSC at room temperature and dehydrated in 70%, 80%, 90%, and 100% ethanol (2 min each). The chromosomal DNA was denatured in 70% formamide in 2× SSC at 70°C for 2 min. The slides were then transferred quickly into cold (−20°C) 70%, 80%, 90%, and 100% ethanol (2 min each) and air-dried. The DNA probe was mixed with a hybridization solution (1.25×) that was prepared as follows: For 10 ml of solution we mixed 6.25 ml of deionized formamide (100%) with 0.625 ml of 1 M Hepes buffer (pH 7.0, sodium salt), 0.125 ml of 100× Denhardt's solution (2% Ficoll/2% polyvinylpyrrolidone/2% bovine serum albumin), 1.875 ml of 20× SSC, 0.525 ml of denatured salmon sperm DNA (10 mg/ml), and 0.188 ml of yeast RNA (in TE). Four milliliters of the 1.25× hybridization solution was then mixed with 1 ml of 50% dextran sulfate. The radioactive NDF probe was mixed with 10% dextran sulfate hybridization mixture at 10^7 cpm/ml, and 0.1 ml of the resulting mixture was used per slide after heat denaturation. The slides were incubated in a moist chamber at 37°C for 16 hr. They were then rinsed in five changes of 50% formamide in 2× SSC at 42°C, followed by five changes of 2× SSC at 42°C (2 min each) and three changes of 0.1×

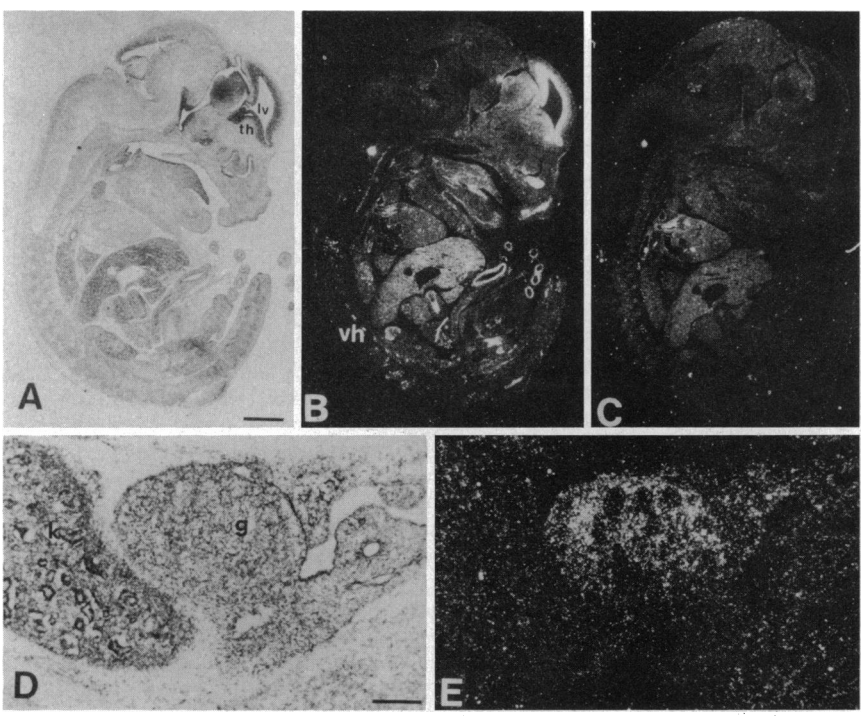

FIG. 1. Localization of NDF transcripts in 14.5-dpc mouse embryos. *A–C* are midsagittal sections; *D* and *E* are sections through the kidney and genital ridge. Shown are bright-field hematoxylin-eosin light green stain (*A* and *D*), and dark-field hybridization with an antisense probe (*B* and *E*) or with a sense probe (control, *C*). Abbreviations: lv, lateral ventricle; th, thalamus; vh, ventral horn of the spinal cord; g, genital ridge; k, kidney. [Bars = 1 mm (*A–C*) or 150 μm (*D* and *E*).]

SSC (all at 42°C). Finally, the slides were dehydrated in 70%, 80%, 90%, and 100% ethanol. For autoradiography the slides were dipped in Kodak nuclear track emulsion (NTB/2) that was diluted 1:1 in water. After 7–21 days at 4°C the slides were developed in Kodak Dektol developer (diluted 1:1 with water) for 2 min at 15°C and transferred to Kodak fixer for 5 min. Following fixation the slides were rinsed in running water for 10–20 min and air-dried.

Chromosome Identification. To visualize G and R banding patterns we used the FPG method (25). Slides were stained in freshly prepared Hoechst 33258 (Sigma) solution (1 μg/ml in 2× SSC) for 15 min. They were then flooded with 2× SSC and exposed under a long-wave UV lamp (Sylvania, GTE) for 1 hr at a distance of 2–5 cm, rinsed in water, and air-dried. Each slide was then stained for 10–15 min in a 7% Giemsa solution (Merck) prepared in phosphate buffer (pH 8.0).

RESULTS

Previous analyses of NDF expression by Northern blotting indicated widespread transcriptional activity of the gene in derivatives of all three embryonic germ layers (18, 19). To precisely localize cells that contain NDF transcripts, we cloned a portion of the murine homolog of NDF, by using the polymerase chain reaction on cDNA (see *Materials and Methods*), and used it as a probe on cryotome sections of 14.5-dpc mouse embryos. This stage of embryonic development was selected because it represents a late step in the process of organogenesis. Fig. 1 shows sagittal sections. Comparison of the bright-field view (Fig. 1*A*) with the dark-field view obtained with an antisense probe of NDF (Fig. 1*B*) or the sense probe that was used for control (Fig. 1*C*) demonstrates that the gene is transcribed at a few specific locations.

The most prominent site of NDF expression is the cortical neuroepithelium that lines the lateral ventricles of the brain (Fig. 1*B*). Less accumulation of NDF transcripts was visible in the brain above the developing thalamus. Other locations of NDF transcripts included the intestines, dorsal ganglia (in a distinct punctate distribution along the spinal cord), the adrenal, the differentiating genital ridge, and the liver (Fig. 1*B*). The autoradiographic signal seen above the atrium of the developing heart is present also in the sense control (Fig. 1*C*) and probably resulted from the birefringence of red blood cells.

More detailed analysis of NDF transcription at selected localizations was performed at higher magnification. The mesenchyme of the genital ridge was relatively rich in NDF transcripts (Fig. 1 *D* and *E*). Transverse sections of the head (Fig. 2 *A* and *B*) demonstrated that the cortical expression corresponded mostly to the deeper layer of the neuroepithelium which lines the lateral ventricles. Adjacent to this layer is a second layer of cells that contains considerably less NDF mRNA. Clonal analysis of neuronal precursor cells has suggested that the differentiation of cortical neurons starts in cell layers that line the ventricular space, and that the cells radially migrate from this location to their final positions within a system of vertical columns in the cortex that share functional properties (26). The stepwise distribution of NDF in the vertical layers of the cortex may be related to this process of differentiation and migration. Additional analysis will be needed to clarify whether NDF-expressing cells are neuronal or glial.

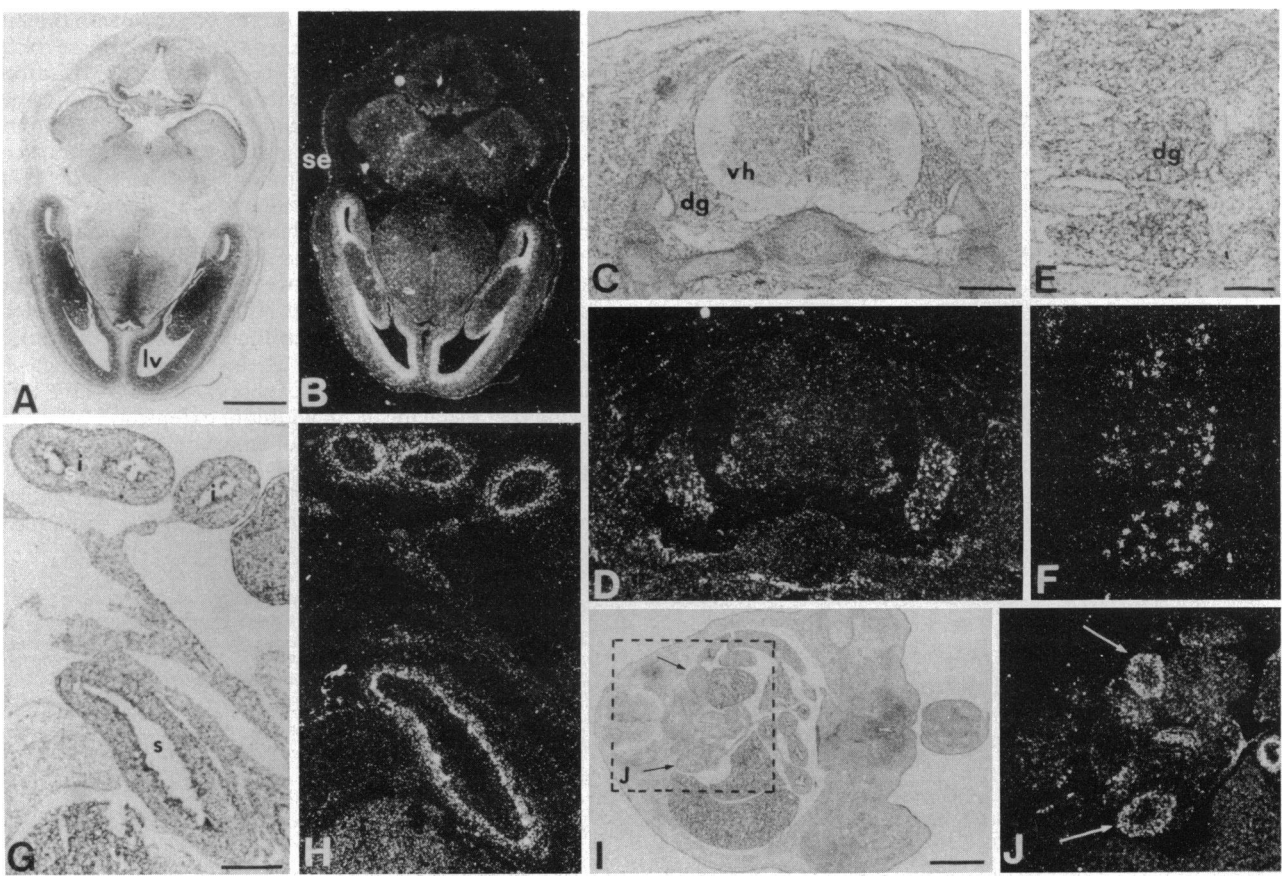

FIG. 2. Localized expression of NDF. Transverse sections of 14.5-dpc mouse embryos are shown in high magnification as pairs of bright-field (*A*, *C*, *E*, *G*, and *I*) and dark-field (*B*, *D*, *F*, *H*, and *J*) micrographs. The boxed area in *I* is represented in *J* and the arrows indicate the adrenal cortex. Abbreviations: dg, dorsal ganglia; i, intestine; lv, lateral ventricle; s, stomach; se, surface ectoderm; vh, ventral horn of the spinal cord. [Bars = 1 mm (*A* and *B*), 300 μm (*C* and *D*), 150 μm (*E–H*), or 500 μm (*I*).]

Other spatial localizations of NDF transcripts may be related to the development and function of the central and peripheral nervous system. For example, transverse (Fig. 2 *C* and *D*) and sagittal (Fig. 2 *E* and *F*) sections of the spinal cord demonstrated that NDF was expressed in specific neurons of the ventral horns of the spinal cord and in the corresponding dorsal ganglia. The labeling in the ventral horns could be seen also in sagittal sections of the whole embryo, and it displayed a longitudinally segmented pattern of punctate appearance corresponding to ventral roots and the dorsal root ganglia (Fig. 2*F*). Interestingly, the distribution of the *neu*-encoded receptor, as determined with specific antibodies, parallels the localization of NDF in the dorsal root ganglia (6).

In situ hybridization of the radiolabeled probe in the intestines and the adrenal cortex was also indicative of NDF expression in the peripheral nervous system. Accumulation of NDF transcripts within the muscular layer of the intestine and the stomach was observed (Fig. 2 *G* and *H*). This localization is in accordance with the distribution of the myenteric ganglia. The adrenal cortex, which is a secretory organ connected with the peripheral nervous system, also showed abundant expression of NDF (Fig. 2 *I* and *J*, arrows).

NDF expression was also detectable in a distinct cellular layer of the dermis (Figs. 1*B* and 2*B*), in the liver (Fig. 1*B*), and in a sheet of cells that lay immediately ventral to the vertebral body (Fig. 2 *C* and *D* and Fig. 1*B*). The identity of these cells remains unknown.

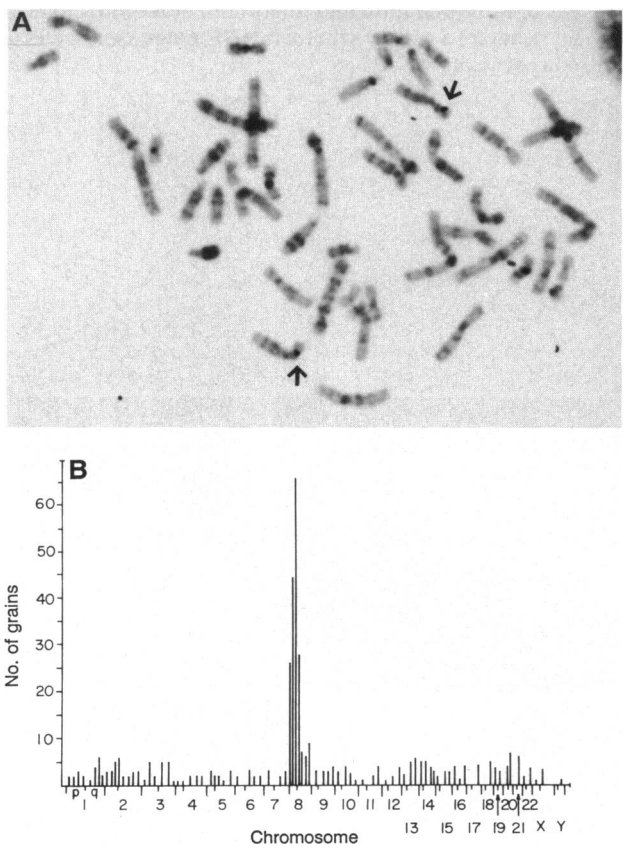

FIG. 3. Localization of the NDF gene to chromosome 8. (*A*) Autoradiograph of metaphase chromosome spread of human peripheral lymphocytes hybridized with a ^{3}H-labeled probe of human NDF. Localization of silver grains to the short arm of both chromosomes 8 is indicated by arrows. (*B*) Quantitative analysis of the distribution of silver grains on 180 normal male metaphases. The chromosomes and their arms (q and p) are represented on the abscissa and the respective number of grains along each chromosome is shown on the ordinate.

Chromosomal mapping of the NDF gene was performed with a 1.8-kb cDNA probe that was labeled with tritiated nucleotides. *In situ* hybridization of the probe to 180 metaphase spreads detected 415 silver grains upon autoradiography. A characteristic autoradiogram is shown in Fig. 3*A*. One hundred sixty-two grains (39%) were concentrated on the centromere and short arm of chromosome 8. No other chromosome displayed a comparable level of labeling, as can be inferred from the histogram shown in Fig. 3*B*. High-resolution localization of the silver grains to specific chromosomal bands showed maximal hybridization to band 8p12 (52 grains, or 36% of the grains on 8c–8p) and band 8p21 (42 grains, 26%). The distribution of grains in these and other bands of human chromosome 8 is schematically shown in Fig. 4. We conclude that the NDF gene is localized to a single chromosomal site within bands 8p12–p21.

DISCUSSION

The aim of these studies was to obtain information that will guide future investigation of the physiological role of a recently discovered growth-regulatory protein. The predominant localization of NDF transcripts to the central and peripheral nervous system unexpectedly raised the possibility that the factor is involved in neuronal functions. In addition, chromosomal mapping of the gene to 8p12–p21 may suggest an association with certain genetic disorders.

The pattern of distribution of NDF transcripts in 14-dpc mouse embryos is similar to the tissue-specific expression of the *neu* gene in midgestation rat embryos (6). Most striking is the coexpression in dorsal root ganglia and in the periphery of brain ventricles (Fig. 1 and ref. 6). Although the precise relationship between these patterns of expression remains to be determined, it is noteworthy that the β-type platelet-derived growth factor (PDGF) receptor and the B isoform of the cognate ligand are transcribed at juxtaposed sites during placental development (27). Similarly, the α receptor and its cognate ligand, PDGF-AA, are appositionally transcribed during embryonic development (28), as are colony-stimulating factor 1 and its *fms*-encoded receptor (29). In this respect, the patterns of distribution of transcripts of NDF and its presumed receptor appear to differ, as they apparently colocalize. It is relevant, however, that receptor–ligand relationships between Neu and NDF have not been unequivocally established: The occurrence of heterodimerization of Neu and EGF receptor (30, 31) leaves open the possibility that NDF affects tyrosine phosphorylation of p185^{neu} through an indirect mechanism. In addition, extension of the expression analysis to other stages of embryonic development may reveal different patterns of NDF distribution.

Interestingly, the *neu* gene is transcribed in embryonic brain but not in adult brain (6). This may be correlated with

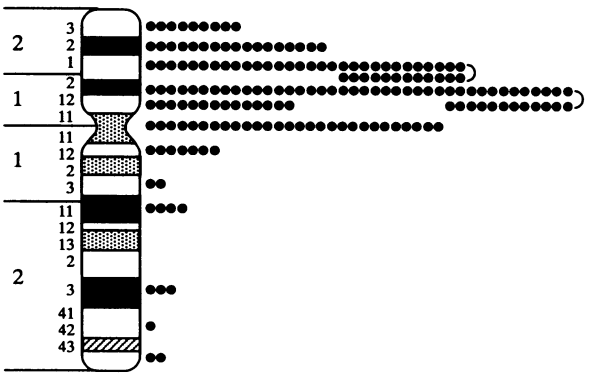

FIG. 4. Idiogram illustrating the distribution of silver grains on the human G-banded chromosome 8.

the period of susceptibility of *neu* to oncogenic activation by a carcinogen (5). From a biochemical point of view the effect of the transforming mutation of *neu* is equivalent to ligand activation of the Neu receptor (9, 12). Conceivably, the period of susceptibility to carcinogenesis and its specificity to neurogenic tumors may reflect a temporal and spatial peak of activity of the endogenous ligand. This possibility is consistent with the predominance of NDF expression in the nervous system and raises the intriguing possibility that the factor has a neuronal role. It may be relevant that *neu* mRNA, as well as the transcript of *trk*, an established receptor for a neurotrophic factor, was increased in adult sciatic nerves undergoing Wallerian degeneration (32).

NDF expression in other, non-neuronal, embryonic tissues is in general consistent with Northern blot analyses (18, 19). Our survey of cultured cell lines so far has indicated that epithelial cells rarely express the gene but that embryo-derived fibroblasts do express it at low levels that undergo remarkable increase upon transformation with the *ras* oncogene (18). This observation is consistent with the detection of NDF transcripts in the dermis (Fig. 1). Although our resolution was insufficient to determine the identity of the dermal cell layer, we assume that it corresponds to the basal layer, which was shown to express $p185^{neu}$ (6).

Mapping of the NDF gene to the short arm of chromosome 8 at bands p12–p21 localized the gene to the vicinity of a few known genes such as those encoding tissue-type plasminogen activator (8p12), fibroblast growth factor receptor 1 (*flg/flt2*; 8p12–p11.2) ankyrin 1 (8p11.2), neurofilament (8p21), and glutathione reductase (8p21.1). More important, recent genetic linkage analysis mapped the mutation of Werner syndrome to 8p12–p11 (33). Individuals affected by this rare autosomal recessive disease display many symptoms of premature aging, but the primary genetic defect remains unknown (34). Interestingly, the phenotype affects mostly the function of ectodermal and mesodermal tissues and includes retarded growth, atrophy and hyperkeratosis of the skin, cataracts, and, often, mild diabetes. However, hematopoietic and lymphoid systems appear normal. It may be significant that neither *neu* nor NDF is expressed in the latter tissues. The possibility that the Werner syndrome mutation affects a growth-regulatory molecule such as NDF is compelling and could explain the pleiotropism of the syndrome. Consistent with a defect in growth regulation, skin fibroblasts of Werner syndrome patients display retarded growth parameters when cultured *in vitro* (35).

Deletions within the short arm of chromosome 8 were reported in 50% (36) or 90% (37) of human colorectal tumors or cell lines, respectively. On the other hand, an extra chromosome 8 (trisomy 8) is by far the most frequent chromosomal aberration in acute myeloid leukemia (38). The relevance of these observations to NDF and to the presumed involvement of the *neu* gene in human adenocarcinomas (39) will have to be addressed by future studies.

We thank Barry Sugarman for communication with the Genome Database and Miriam Fagan for typing the manuscript. This work was supported in part by National Institutes of Health Grant CA 51712, by the Laub Center for Oncogene Research, and by the Pasteur–Weizmann Joint Research Program. Y.Y. is a recipient of a Research Career Development Award from The Israel Cancer Research Fund.

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